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Enterovirus Heart Disease of Adults: A Persistent, Limited Organ Infection in the Presence of Neutralizing Antibodies

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Detection of enterovirus RNA in endomyocardial biopsies (EMB) by reverse transcription/polymerase chain reaction (RT-PCR) is currently the preferred diagnostic procedure in suspected enterovirus heart disease (EHD), which can present clinically as myocarditis, dilated cardiomyopathy (DCM), and arrhythmogenic right ventricular cardiomyopathy (ARVC). EMB and peripheral blood mononuclear cells (PBMC) of 44 patients with suspected EHD were examined by nested RT-PCR to investigate whether the myocardial enterovirus infection is limited to the heart or is generalized. Enterovirus RNA was detected in EMB, but not in PBMC, of 8 patients (3 of these suffered from ARVC), whereas EMB of 16 controls and PBMC of 45 controls were negative. In addition, enterovirus RNA was demonstrated in PBMC, but not in EMB, of a single patient with suspected EHD. A high sequence homology of the amplicons to coxsackievirus B3 was demonstrated in 7 patients, and to coxsackievirus B2 in two patients. In order to evaluate whether the myocardial enterovirus infection was acute or persistent, neutralization and complement fixation tests were performed for antibodies against the serotypes indicated by the nucleic acid sequences. Neutralizing antibodies were detected in the sera of all 9 patients, but complement fixing antibodies were demonstrated only in one EHD patient and in the patient positive for enterovirus RNA in PBMC. In conclusion, the molecular and serological data demonstrate that CVB3 predominates as cardiotropic enterovirus, and that the enterovirus replication is limited to the heart in EHD. Serological results support the hypothesis of myocardial enterovirus RNA persistence in spite of neutralizing antibodies. ***J. Med. Virol. 53:196–204, 1997.***

KEY WORDS: myocarditis; dilated cardiomyopathy; arrhythmogenic right ventricular cardiomyopathy; coxsackievirus; myocardial infection

INTRODUCTION

Acute and chronic myocarditis were described early in enterovirus, especially coxsackievirus, epidemics [Abelmann 1973, Woodruff 1980]. The detection of coxsackie B specific IgM and IgG antibodies in patients with myocarditis supported the etiological significance of enteroviridae in myocarditis [Cambridge et al. 1979, Grist and Bell 1974, Muir et al. 1989, Vickersfors et al. 1988], but propagation of enterovirus from heart tissue was successful only in a few cases [Woodruff 1980, Abelmann 1973, Longson et al. 1969, Sutton et al. 1967, Maller et al. 1967]. Subsequently, evidence of enterovirus replication in the affected myocardium was achieved using slot-blot and in situ nucleic acid hybridization in EMB of patients suffering from myocarditis or dilated cardiomyopathy (DCM), which may be a sequel of myocarditis. 15 to 43% of the patients were shown to be positive for enterovirus RNA, whereas EMB of control patients suffering from various other heart diseases were negative [Bowles et al. 1986, Bowles et al. 1989, Easton and Eglin 1988, Hilton et al. 1993, Kandolf and Hofschneider 1989, Tracy et al. 1990, Why et al. 1994].

Subsequently, interest was focused on the rapid and sensitive method of RT-PCR to demonstrate enterovirus RNA in the myocardium. Various different oligo-

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nucleotide primers were proposed, most of them specific for the highly conserved 5′non-translated region (5′NTR) of the enterovirus genome [Andreoletti et al. 1996, Chapman et al. 1990, Jin et al. 1990, Kämmerer et al. 1994, Rotbart 1990, Schwaiger et al. 1993]. Recently, enterovirus RNA was detected in EMB of a few patients suffering from arrhythmogenic right ventricular cardiomyopathy (ARVC), which may be, similar to DCM, a sequel of a myocarditis in some cases [Giacca et al. 1994, Heim et al. 1997]. Therefore, six ARVC patients were included in the present study.

In spite of the detection of enterovirus RNA in the myocardium, the pattern of enterovirus replication and persistence in human myocarditis is still poorly understood. In the mouse model of EHD, enterovirus RNA persistence was detected both in the heart and in lymphocytes of thymus and spleen [Klingel et al. 1992], but no data have been published on enterovirus replication in lymphoid cells in human myocarditis. As human mononuclear leukocytes can be persistently infected with coxsackievirus in vitro [Mateucci et al. 1985], it seems probable that enterovirus RNA may be found in the blood of EHD patients, either because of enterovirus persistence in lymphoid organs and PBMC or as a result of virus shedding from the heart. So far, only a small number of children suffering from myocarditis were tested for blood borne enterovirus RNA by highly sensitive RT-PCR and turned out to be negative [Martin et al. 1994]. However, enterovirus RNA was frequently detected by RT-PCR in the blood of patients with various other enterovirus associated diseases [Clements et al. 1995, Clements et al. 1995, Nairn et al. 1995, Thoren et al. 1992]. Therefore, the aim of our study was to elucidate the presence of enterovirus RNA in the blood of patients with suspected EHD and to search for a correlation with enterovirus RNA in the myocardium.

A second aim of the study was to identify the amplified enterovirus RNA by nucleic acid sequencing in order to permit a serotype directed search for neutralizing and complement fixing antibodies. Thus, it may be possible to overcome the drawbacks of serology, which are associated with the multitude of enterovirus serotypes, after a group specific detection of enterovirus RNA in the heart. Detection of complement fixing antibodies to the myocardial pathogen can indicate an acute or recent infection of the heart whereas absence of complement fixing antibodies may indicate enterovirus RNA persistence. Moreover, detection of neutralizing antibodies may clarify, if enterovirus RNA can persist in the heart in spite of a humoral immune response.

MATERIALS AND METHODS

Study Design, Patients and Control Groups

Forty-four adult patients who presented at the Göttingen University Clinical Center between September 1994 and March 1996 with suspected EHD were prospectively included in this study. EHD was suspected in all patients who (i) fulfilled the diagnostic criteria of dilated cardiomyopathy (34 patients) according to the definition of the world health organization [WHO 1980], or (ii) fulfilled the diagnostic criteria of right ventricular cardiomyopathy/dysplasia (ARVC) (6 patients) as proposed by an international task force recently [McKenna et al. 1994], or (iii) had a positive clincal myocarditis score (4 patients) as proposed by Dec et al. [1985], which was confirmed by results of histopathology [Aretz 1987]. Seven of 34 patients, who suffered clinically from DCM, were diagnosed histopathologically either as myocarditis, borderline myocarditis, or displayed a picture similar to healed myocarditis as described in the Dallas criteria [Aretz 1987]. Mean age of the patients was 51 years (SD = 11.9), 10 were female. The following diagnostic procedures were performed on entry of the study: A clinical and comprehensive anamnestic work up with determination of duration of preclinical symptoms, chest radiography, electrocardiography, echocardiography, standard laboratory tests, and left ventricular catheterization (Judkins technique, right ventricular catherization in case of ARVC patients). 5 endomyocardial biopsies were taken from every patient (Cordis long sheet bioptome, 5.4 F or 7F) for histopathology, molecular diagnosis of EHD by RT-PCR and nucleic acid sequencing. 30 ml blood were taken for molecular detection of enterovirus viremia, virus propagation and enterovirus serology simultaneously to endomyocardial biopsy. As most patients were discharged quickly after diagnostic endomyocardial biopsy, only one blood sample of each patient was taken. Blood samples of 45 patients with coronary artery disease served as control group for serology and detection of enterovirus RNA by RT-PCR to determine the frequency of non-myocardial enterovirus infections in the same geographic area and period of time (control goup A). Patients of control group A did not differ demographically, socioeconomically, and in sex distribution (12 female) from the patients with suspected EHD, but were older than patients with suspected EHD (mean age 66.4, SD = 10.9 vs. 50.6, SD = 11.9 years). Patients of control group A were only subject to medical examinations related to diagnosis and treatment of coronary artery disease and did not undergo EMB. Although this study was not designed primarily to demonstrate the significance of enterovirus RNA detection in the myocardium of patients suffering from myocarditis, dilated cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy, a second control goup (B) was included. EMB of 16 patients (mean age 49 years, 6 female) who suffered from various other heart diseases not associated with enterovirus infections, e.g. amyloidosis (3 patients), alcoholic cardiomyopathy (1 patient), idiopathic ventricular arrhythmias (4 patients), hypertrophic cardiomyopathy (2 patients), hypertensive cardiomyopathy (4 patients), Morbus Becker (1 patient), restrictive cardiomyopathy (1 patient), were tested for enterovirus RNA.

Clinical Specimens

All EMB were divided immediately under strictly sterile conditions into two roughly equal parts, one for histopathology, which was fixed according to standard procedures with formaldehyde, the other part was frozen in liquid nitrogen and stored at −80°C for RT-PCR. Total RNA was extracted from endomyocardial myocardial tissue after grinding of frozen tissue under liquid nitrogen. PBMC were isolated from 20 ml EDTA treated blood by ficoll-paque density gradient centrifugation (Pharmacia, Sollentuna, Sweden). Total RNA was extracted both from EMB and PBMC by the guanidiniumisothiocyanat/acid phenol method as described by Chomczynski [1987], but adapted for use with smaller amounts of tissue by reducing all volumes to ¼ of the original protocol. Purified RNA was dissolved in 30 μl diethylpyrocarbonate treated water, RNA concentration was determined by photometry at 260 and 280 nm. The presence of reverse transcriptase or Taq-polymerase inhibitors was excluded in each RNA sample by successfully performing a β-Actin RT-PCR as described by the manufacturer of the control RT-PCR kit (Clontech, Palo Alto, CA). In addition, RNA was extracted from 140 μl serum of every patient using a silica-membrane technique (HCV virus RNA kit, Qiagen, Hilden, Germany) and used for enterovirus RT-PCR.

cDNA Synthesis and Nested PCR

The reverse transcription reaction was carried out using 1 pmol enterovirus specific primer, Coxprim2 (ATGAAACCCACAGGCAC), and Superscript 2 RNAse-negative Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) in the buffer delivered with the enzyme, containing 0.5 mM (each) deoxynucleotides triphosphate, and 1 mM dithiothreitol in a total volume of 30 μl at 48°C for 60 minutes. 0.5 μg RNA was used for reverse transcription in case of EMB and PBMC, and 16 μl of the RNA containing eluate in case of the serum samples, respectively. All oligonucleotide primers were obtained from MWG-Biotech (Ebersberg, FRG). Nested PCR was done basically as described by Kämmerer [1994] under strict precautions to avoid product carry-over and contamination [Kwok and Higuchi 1989]. In contrast to the published protocol [Kämmerer et al. 1994], a "hot start" was performed using 25 μl Biowax (Biozym, Hess.-Oldendorff, FRG), and the reaction buffer was slightly modified to 10 mM Tris-HCl pH 9.2, 3.5 mM $MgCl_2$, 25 mM KCl, containing 0.25 mM of each deoxynucleotidetriphosphat, 2.5 U Taq Polymerase (Life Technologies, Gaithersburg, MD), and 0.8 pmol of each primer in a total volume of 80 μl. Primers for the PCR were Coxprim2 and Coxprim1 (ACCTTTGTACGCTGTT), and for the nested PCR Coxprim 3 (AAGCACTTCTGTGTTTCCC) and Coxprim 4 (AGGAGGCCGGGGACTTA) resulting in a 297 bp product corresponding to the nucleotides 166 to 463 of the 5′ non translated region of CVB3. PCR products were analyzed by electrophoresis on 2% agarose/TAE gels stained with ethidium bromide. RNA samples extracted from CVB3 infected and mock infected Vero cell cultures served as internal positive and negative controls, respectively.

Nucleic Acid Sequencing

Nested PCR products were purified by a preparative 2% agarose gel electrophoresis. Bands were extracted from the agarose by a silica membrane method (Qiaquick gel extraction kit, Qiagen, Hilden, FRG). Forty fmol of the purified DNA were sequenced directly (without cloning) with a cycle-sequencing protocol (dideoxy-mediated chain-termination, fmol sequencing kit, Promega Madison, WI). DNA was labeled with biotinilated sequencing primers Coxprim3 (or Coxprim4, respectively) and blotted on a nylon membrane with a direct blotting device (GATC, Konstanz, FRG). Visualization was done by chemo-luminescence with CSPD using the Seq-Light kit (Tropix, Bedford, MA). Determined sequences were aligned to enterovirus sequences available from the European Bioinformatics Institute (EBI, Huxton Hall, UK) using the DNASIS v 6.0 software and a multiple alignment software (Corpet 1988). In addition, five amplicons of the internal positive control reactions (CVB3, Nancy strain) were sequenced.

Nucleic Acid Sequencing of Coxsackievirus B2

Coxsackievirus B2 (CVB2, Ohio-1 strain, communicated by R. Kandolf, Martinsried, FRG) was propagated on Vero cells and harvested from the cell culture supernatants at 90% CPE. After centrifugation for 15 min at 15000 g and 4°C, RNA was extracted with the Qiagen viral RNA kit (Qiagen Hilden, FRG]. Reverse transcription was performed as described above with the enterovirus specific primer RB-1 (ATTGTCACCATAAGCAGCCA, Rotbart 1990). CVB2 cDNA was amplified with the primers RB-1 and a second enterovirus specific primer (CGGTACCTTTGTGCGCCTGT, corresponding to nucleotides 63-83 of the CVB3 genome), amplitaq gold (Perkin Elmer Cetus, Norwalk, CT), and PCR optimization buffer #4 (Stratagene, La Jolla, CA). Forty cycles of 30 s at 55°C, 45 s at 73°C, and 30 s at 94°C were performed after a preincubation of 5 min at 94°C. The PCR product was purified as described above. 400 fmol of the purified DNA were sequenced using the GATC-Biocycle sequencing kit (GATC, Konstanz, FRG) with biotinilated dideoxynucleotide chain terminators and the same oligonucleotide primers as used in DNA amplification. Blotting and visualization of the reaction products were done as described above.

Serology

Sera of enterovirus RT-PCR positive patients were tested for neutralization activity against the enterovirus serotype indicated by the results of nucleic acid

sequencing. In addition, each serum (all patients with suspected EHD and control group A) was tested for neutralizing activity against CVB3. Neutralization tests against CVB3 were done according to a standard protocol in 96-well microtiter plates [Chernesky et al. 1996]. Briefly, serial two-fold dilutions of sera with Dulbecco's modified Eagle's minimal medium (DMEM) containing 5% FBS were incubated with 10^4 plaque-forming units (PFU) of transfection derived CVB3 Nancy strain [Kandolf et al. 1985] for 2 h at room temperature. In addition to positive and negative control sera, a dilution of the neutralizing monoclonal antibody (MAB) 948 (Chemicon, Temecula CA) was used as a positive control. 10^4 Vero cells were added to each well and plates were incubated for 36 h at 37°C and 5% CO_2. Protection from cytopathogenic effects was determined by light microscopy and by staining of viable cells with EZ4U (Biozol, Vienna, Austria) and subsequent photometry at 450 nm (reference wavelength 620 nm). Neutralizing tests were concerned positive according to laboratory internal standards if protection from CPE was achieved with serum dilutions of 1:16 or higher. Tests for neutralizing antibodies against coxsackievirus B2 were done similarly with the appropriate enterovirus, control sera and a CVB2 specific neutralizing MAB 946 (Chemicon, Temecula CA) as additional internal positive control. Complement fixation tests were carried out with heat treated (30 min. 56°C) patients' sera according to a standard protocol on 96 well microtiter plates [Chernesky et al. 1996]. Standardized antigen preparations of either coxsackie B1, B2, B3, B4, B5, B6, or A9 and control antigen preparations from mock infected cells were used (Institut Virion, Rüschlikon, Switzerland). Adequate positive and negative control sera were purchased from the manufacturer of the antigen preparations and complement fixation tests were considered positive according to laboratory internal standards if a serum dilution of 1:8 or higher was reactive.

Tissue Culture

Virus isolation from patients' sera was attempted basically as described in a standard protocol [Wiedbrauck and Johnston 1993]. In modification of the standard protocol, four different cell lines Vero (ATCC CCL 81), HeLa (ATCC CCL 2.2), Hep2 (ATCC CCL-171), and MRC5 (ECAAC #84101801) were used. Briefly, 100 μl serum was incubated for 1 h with each cell line, cultures were washed with PBS, and fresh cell culture medium was added. Cells were propagated for a period of 14 days (including two times passaging by trypsinization) and examined for typical CPE every third day.

RESULTS

Enterovirus Detection

Enterovirus RNA was demonstrated by nested RT-PCR in EMB of 8 patients (of total 44 patients) with suspected EHD (Fig. 1). Three EHD patients suffered clinically from ARVC, the remaining 5 patients had the clinical diagnosis DCM, but in 3 of these 5 patients

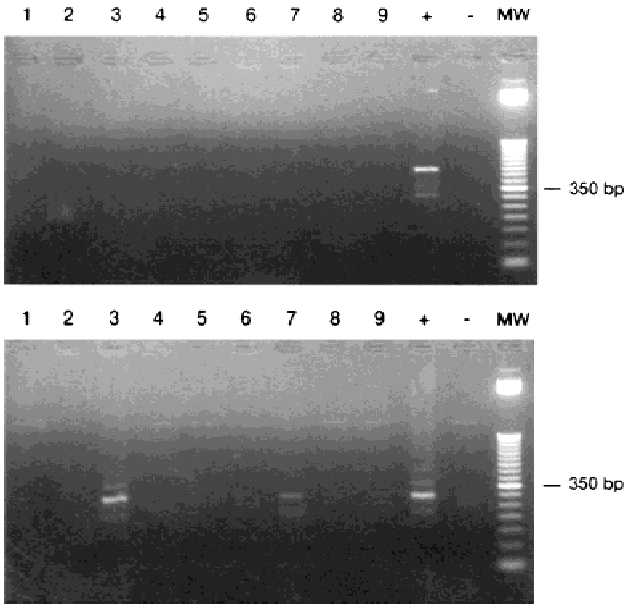

Fig. 1. Ethidium-bromide stained agarose gels with RT-PCR products. Lanes 1–9: samples of patients with suspected EHD. Lanes 3 and 7 correspond to EHD patients #1 and #2, respectively. +: positive control, −: negative control, MW: molecular weight standard (50 base-pair (bp) ladder). Upper gel: first PCR, lower gel: nested PCR.

histopathology revealed borderline myocarditis or a histopathological picture typical for healed myocarditis (Table I). Interestingly, no enterovirus RNA was detected by nested RT-PCR in PBMC and sera of all patients positive for enterovirus RNA in the myocardium. In addition, virus isolation from all 89 serum samples of patients with suspected EHD and control group A had negative results. Enterovirus RNA was demonstrated by RT-PCR (nested) in PBMC, but not in EMB and serum of a single patient (#4) with suspected EHD. Detailed data on all enterovirus positive patients are depicted in Table I.

The 35 patients with suspected EHD, who were negative for enterovirus RNA in RT-PCR, did not differ significantly from the 9 enterovirus RNA positive patients in relation to mean age (50.3 vs. 52.4 years, $P = 0.49$, t test), sex (7 of 28 female vs. 3 of 9 female, $P = 0.39$; Chi-Square) or mean duration of cardiac symptoms (20.8 vs. 20.7 months, $P = 0.74$; t test). All 45 subjects of control group A were negative for enterovirus RNA by nested RT-PCR both in PBMC and sera. Besides, all EMB of control group B (16 subjects with various other cardiac disorders, but without suspicion of enterovirus heart disease) were negative for enterovirus RNA.

Nucleic Acid Sequencing and Multiple Alignments

As presumed, nucleic acid sequencing confirmed that enterovirus RNA was amplified from EMB or PBMC of the patients. Sequence data were submitted to the EBI. Accession numbers are as follows: amplicon of patient #1: Y11010, amplicon of patient #2: X97978, amplicon

TABLE I. Clinical, Histopathological and Virological Characteristics of Enterovirus RNA Positive Patients

Patient #	Age (years)	Sex	History duration (months)	Clinical diagnosis	Histopathology	RT-PCR[a] EMB/PBMC	CFT (titer)	NT (titer) CVB3/CVB2
1	44	f	3	DCM	DCM	CVB3/neg	neg	>512/nd
2	54	f	24	ARVC	ARVC	CVB2/neg	neg	8/>512
3	54	m	108	ARVC	ARVC	CVB3/neg	B1(8)	32/nd
4	37	m	36	DCM	healed MC[a]	neg/CVB3	B1(8), B3(8), B4(16), B5(8), B6(8), A9(8)	256/nd
5	54	m	6	DCM	DCM	CVB3/neg	neg	32/nd
6	70	f	3	ARVC	ARVC	CVB3/neg	B3 (8)	>512/nd
7	74	m	13	DCM	healed MC[a]	CVB3/neg	n.e.	256/nd
8	31	m	<1	DCM	DCM	CVB2/neg	neg	256/>512
9	54	m	1	DCM	borderline MC	CVB3/neg	n.e.	128/nd

[a]Histopathology similar to healed myocarditis as described in the Dallas Criteria (Aretz 1987).

of patient #3: X97977, amplicon of patient #4: Y11011, amplicon of patient #5: Y11012, amplicon of patient #6: Y11013, amplicon of patient #7: Y11014, amplicon of patient #8: Y11015, amplicon of patient #9: Y11016. The amplicons of patient #1, #3, #4, #5, #6, #7, and #9 demonstrated a high homology (98 to 99%) to the 5′non translated region of CVB3. In these 7 amplicons, the highest homology prevailed to the previously published sequence of cardiotropic CVB3 Nancy strain (EBI # M33854) and a slightly lower homology to the cardiotropic Woodruff strain of CVB3 (U57056) (Fig. 2). Six amplicons of the positive control (cell culture derived CVB3 Nancy strain) were sequenced. These were all identical to the published CVB3 sequence (EBI #M33854), thus demonstrating the reliability of the applied method.

The amplicons of the remaining two patients (#2 and #8) had a considerable lower homology to CVB3 (both 90%) and all other published enterovirus sequences. For example, the amplicon of patient #2 had a 87% homology to coxsackie A9, B1 and B5, and the amplicon of patient #8 was 90% homologous to coxsackie B1 and 87% to B5. Since CVB2 has been suspected to be cardiotropic as the other coxsackieviruses of subgroup B, but no CVB2 sequence has been published so far, we supposed that the PCR amplicons of patient #2 and #8 may have a high homology to CVB2. Therefore, the 5′NTR of CVB2 was sequenced in part (EBI # Y09512). Alignments of the nucleic acid sequences of the two amplicons from EMB (patient #2 and #8) with the CVB2 sequence (EBI # Y09512) demonstrated the highest homology (96% and 98%, respectively) compared to all other enterovirus sequences.

A 140 bp fragment of the CVB2 sequence was also included in a multiple alignment of all patients' amplicons with the published human enterovirus sequences. Figure 2 depicts a dendrogram of the results of the multiple alignment and clustering. As it was presumed, the sequence of CVB2 is closely related to most other coxsackie and echovirus sequences, but far less homologous to poliovirus and coxsackievirus A21 and A24 sequences. Surprisingly, the amplicon of patient #8 was clustered to the sequence of echovirus 11 by the multiple alignment software. However, only a 140 bp fragment that was resolved in both strands of each amplicon was used for multiple alignment. Calculation of individual homology scores for the 168 bp sequence resolved in both strands of the PCR amplicon of patient #8 with CVB2 and echovirus 11 sequences demonstrated a higher homlogy to CVB2 (96%) than to echovirus 11 (86%).

Serology

Neutralizing antibodies (titer ⩾16) against CVB3 were demonstrated in all 7 patients, who had PCR amplicons highly homologous (>97%) to CVB3 (Table I), but only in 26/45 patients of control group A ($P = 0.044$, Chi-Square). Both patients (#2 and #8), who were positive for enterovirus RNA highly homologous to CVB2, had neutralizing antibody titers >512 against CVB2 (Table I). As CVB3 seemed to predominate in patients with suspected EHD, all patients' sera and all sera of control group A were tested for their CVB3 neutralizing activity. This was more frequently detected in patients with suspected EHD (34/44 serum samples postive) than in control group A (26/45 sera positive; $P = 0.0498$, Chi-Square Test).

Only two of seven patients (#4 and #6, Table I), who were positive for CVB3 by nested RT-PCR and nucleic acid sequencing, had complement fixing antibodies against CVB3. Two CVB3 RNA positive patients were not evaluable in complement fixation tests due to reactivity with the control antigen preparation. Both CVB2 RNA positive EHD patients were negative in CVB2 complement fixation tests. Detailed data on complement fixing antibodies in enterovirus RNA positive patients are depicted in Table I.

Although complement fixation tests are not sufficient to prove an acute infection with a single serum sample [Melnick 1996], the frequency of recent infections with potentially cardiotropic enterovirus during the study period may be evaluated better by complement fixation tests than with neutralization tests. All sera (suspected EHD group and control group A) were tested for complement fixing antibodies against coxsackievirus B1–B6 and A9. 13 of 41 patients with suspected EHD were positive for complement fixing antibodies against at least one of the serotypes tested. Serum samples of 3 patients with suspected EHD (2 of these were CVB3 RNA positive) were not evaluable in

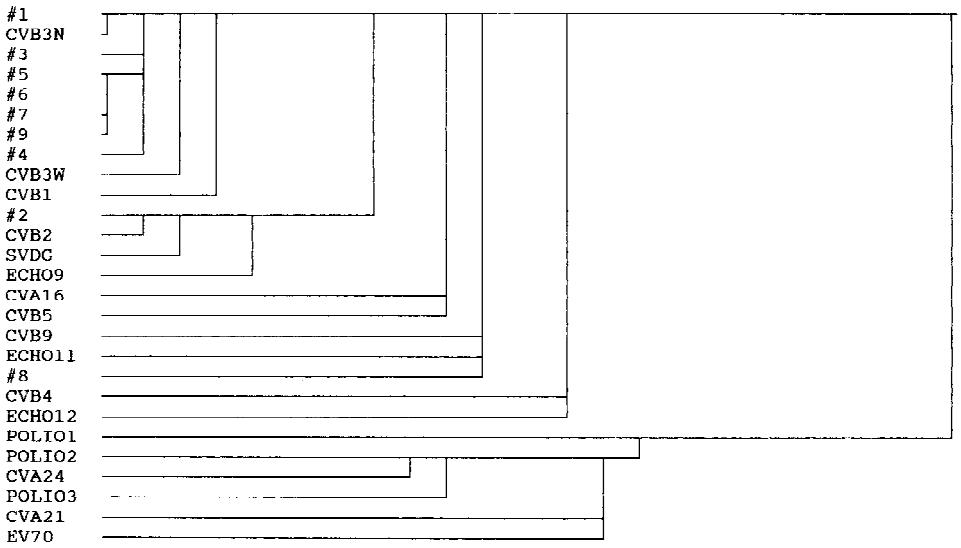

Fig. 2. Hierarchical clustering of 140 bp fragments (corresponding to nucleotides 234–373 of CVB3) of patients #1–#9 with published sequences (EBI accession #) of coxsackievirus B3 (CVB3N: M33854, CVB3W: U57056), coxsackievirus B1 (CVB1: M16560), coxsackievirus B2 (CVB2: Y09512), coxsackievirus B6 (SVDG: D00435), echovirus 9 (ECHO9: X92886), coxsackievirus A16 (CVA16: U05876), coxsackievirus B5 (CVB5: X67706), coxsackievirus A9 (CVA9: D00627), echovirus 11 (ECHO11: X80059), coxsackievirus B4 (CVB4: X05690), echovirus 12 (ECHO12: X79047), poliovirus 1 (POLIO1: V01148), poliovirus 2 (POLIO2: M12197), coxsackievirus A24 (CVA24: D90457), poliovirus 3 (POLIO3: K01392), coxsackievirus A21 (CVA21: D00538), enterovirus 70 (ENTERO70: D00820).

complement fixation tests due to reaction with the control antigen preparation. The frequency of complement fixing antibodies against at least one of the enterovirus serotypes tested was almost identical in control group A (13 of 42 sera positive, 3 sera not evaluable) compared to patients with suspected EHD. In addition, there was no significant difference between titers of complement fixation tests of patients with suspected EHD compared to control group A. Fourteen of the 26 positive sera (including sera of patients with suspected EHD and control group A) had complement fixing antibodies against more than one serotype. Most frequently, complement fixing antibodies were detected against coxsackievirus A9 (6/41 in patients with suspected EHD vs. 8/42 in control group A), coxsackievirus B6 (6/41 vs. 7/42), and coxsackievirus B1 (6/41 vs. 1/42), whereas complement fixing antibodies against CVB3 were detected only in 2/41 patients with suspected EHD (both were CVB3 RNA positive), and 4/42 patients of control group A. In summary, this indicates that recent infections with coxsackievirus A9 and B6 were more frequent than infections with CVB3 during the study period, but obviously not associated with infections of the heart.

DISCUSSION

In the present study, enterovirus RNA was detected in 6 of 34 DCM patients (roughly 18%) compared to 0 to 49% in previous studies [Bowles et al. 1989, Kandolf and Hofschneider 1989, Jin et al. 1990, Liljeqvist et al. 1993, Kämmerer et al. 1994, Satoh et al. 1994, Figulla et al. 1995]. Concerning these varying results on enterovirus detection in DCM patients, it should be kept in mind that DCM is so far defined as an idiopathic disorder which may be a sequel of enterovirus myocarditis in some patients, may have a genetic etiology in other patients, or have another so far obscure etiology [Mestroni et al. 1994, WHO 1980]. Nevertheless, enterovirus RT-PCR should be applied to EMB of every DCM patient in order to elucidate one possible etiology of this disorder.

In contrast to all nucleic acid hybridization studies and most RT-PCR studies, a few RT-PCR studies had similar frequencies of enterovirus RNA detection in the myocardium of patients with dilated cardiomyopathy and control patients [Giacca et al. 1994, Keeling et al. 1992, Petitjean et al. 1992]. Besides the possibility of PCR product carry over contamination, the inclusion of chronic coronary artery disease patients into the control group [Petitjean et al. 1992, Keeling et al. 1992] may be an explanation for equivalent frequencies of enterovirus RNA detection in the dilated cardiomyopathy group and the control group. Recently, an association of chronic coronary artery disease (but not acute myocardial infarction) with enterovirus infections of the heart was demonstrated in a study using healthy heart donors as an adequate control group [Andreoletti et al., 1996]. Although a recent study demonstrated that group B coxsackieviruses can persistently infect human endothelial cells in vitro [Conaldi et al. 1997], the pathogenetical significance of enterovirus replication in chronic coronary artery disease is still obscure. Patients with coronary artery disease were included in control group A of the present study (enterovirus RNA detection in blood and serology, all negative), but were not included in control group B (enterovirus RNA detection in EMB, all negative).

In spite of the multitude of studies on enterovirus

heart disease, a systematic approach to detect bloodborne enterovirus RNA simultaneously with demonstration of enterovirus RNA in the heart was not attempted so far. Since observations in a mouse model of EHD indicated a widespread enterovirus infection of lymphocytes [Klingel et al. 1992], and human lymphocytes can be easily infected in vitro [Mateucci et al. 1985], we searched for enterovirus RNA in PBMC of patients with suspected EHD by nested RT-PCR. CVB3 RNA was demonstrated in PBMC of a single patient (#4, Table I) with suspected EHD (clinical diagnosis DCM, histopathological diagnosis healed myocarditis). Surprisingly, this patient was enterovirus negative in EMB. This may be due to the well known phenomenon of sampling error of diagnostic EMB if patient #4 suffered really from enterovirus heart disease [Aretz 1987] or, on the other hand, the enterovirus detection in PBMC may be caused by a non myocardial enterovirus infection. As the amplified enterovirus RNA was highly homolgous to CVB3, which predominated as cardiac pathogen in the present and other studies [Nicholson et al. 1995, Khan et al. 1995], we propose that the patient did indeed suffer from EHD. This view is also supported by a histopathological picture similar to healed myocarditis as defined in the Dallas criteria [Aretz 1987], which is quite typical in enterovirus positive DCM, and by the result that the 45 PBMC samples of control group A were enterovirus RNA negative. Hence, enterovirus RT-PCR of PBMC samples in addition to EMB may slightly enhance the sensitivity of enterovirus detection in suspected EHD. On the other hand, an enterovirus infection of PBMC is not frequent in EHD and probably not pathogenetically significant in manifest EHD of adult patients. It is widely accepted that attempts to isolate enterovirus from the blood often fail during enterovirus infections of adults, obviously because viremias are of short duration and appear only early in infections [Melnick 1996]. Our results demonstrate that this is also true in EHD of adult patients, who do not suffer from symptoms of an acute enterovirus infection as for example common cold symptoms, diarrhoea, and fever, even if a highly sensitive nested RT-PCR is used in addition to virus propagation.

Although enterovirus RNA detection is feasible in suspected EHD for several years, it is not known in most patients whether the myocardial enterovirus infection was acute or persistent. A long history of cardiac symptoms before enterovirus RNA detection in EMB indicates a persistent infection of the heart quite frequently [Bowles et al. 1989, Figulla et al. 1995]. Enterovirus persistence in the myocardium was demonstrated in a few patients studied so far by repeating EMB and enterovirus RNA detection after e.g. 6 months [Figulla et al. 1995, Satoh et al. 1996]. On the other hand, EMB is an invasive procedure with a low, but significant risk for the patient, and therefore repeating EMB should be avoided if possible [Starling et al. 1991]. Serological tests hold promise to distinguish acute from persistent enterovirus infections, but detection of IgM or complement fixing antibodies may not succeed after a group specific demonstration of enterovirus RNA in the myocardium due to the multitude of serotypes. The use of heterotypic (enterovirus group specific) IgM ELISA (enzyme linked immunosorbent assays) may solve this problem in part [McCartney et al. 1986, Swanink et al. 1993]. As enterovirus infections have a high incidence [Melnick 1996], it remains still doubtful, whether heterotypic IgM is a result of myocardial enterovirus replication or a result of a concomitant non-myocardial enterovirus infection with another serotype. Sequencing of the enteroviral nucleic acid amplified from the myocardium permits an unambiguous detection of serotype specific antibodies. In the present study, the enteroviral 5′NTR was amplified by RT-PCR and sequenced for identification of the myocardial pathogen. This approach is favourable, because a group specific amplification of enterovirus RNA is achieved [Kämmerer et al. 1994], but, on the other hand, some variability between the capsid-coding region, which includes the serological determinants, and the sequenced 5′NTR can not be completely excluded. After identification of the myocardial pathogen, complement fixation tests were used to search for signs of an acute or recent antigenic stimulus. Negative complement fixation assays in patients positive for enterovirus RNA and neutralization test, which may be positive life-long after an infection, were interpreted as a sign of enterovirus RNA persistence. This approach tends to overestimate the frequency of acute myocardial enterovirus infections, as there may be some cross reactivity of complement fixing antibodies of recent non myocardial enterovirus infections with the pathogen detected in the myocardium [Melnick 1996]. However, only two EHD patients had complement fixing antibodies against the enterovirus, which was demonstrated by RT-PCR and sequencing of the amplicon (Table I), thus indicating an acute or recent infection. On the other hand, a persistent infection was indicated by negative complement fixation tests and positive neutralization tests in 5 of 9 EHD patients, most of these with a long history duration (Table I, two EHD patients were not evaluable in complement fixation tests). Enterovirus RNA detection in the presence of neutralizing antibodies, but without immunological signs of an acute antigenic stimulus, may be caused by an enterovirus persistence similar to restricted RNA replication, which was demonstrated in the mouse model of EHD [Klingel et al. 1992].

Since CVB3 turned out to be the predominant cardiotropic enterovirus in the present study, it was decided to test sera of all patients with suspected EHD and all sera of control group A for CVB3 neutralizing antibodies. These were more often demonstrated in all patients with suspected EHD (34/44), than in control group A having similar demographic and socioeconomic characteristics, but a higher mean age (66.4 years, SD = 10.9 vs. 50.6 years, SD = 11.9 years). However, the slightly significant difference in CVB3 neutralizaing antibodies ($P = 0.0498$, Chi-Square) may

not be explained by the higher mean age of control group A, as neutralizing antibodies tend to persist life long and prevalence increases with increasing age [Melnick 1996]. Indeed, the significance of CVB3 neutralization assays may be underestimated because of the higher mean age of control patients. Therefore, our data on CVB3 neutralizing antibodies are consistent with previous studies which demonstrated neutralizing antibodies against coxsackievirus of subgroup B more frequently in patients with myocarditis and dilated cardiomyopathy than in control subjects, but enterovirus detection in the myocardium was not yet possible then [Grist and Bell 1974, Vikerfors et al. 1988, Cambridge et al. 1979]. However, no association of neutralizing antibodies with end stage DCM was reported by Bowles et al. [1989]. A possible explanation may be that end stage DCM patients failed to react sufficiently to the myocardial enterovirus infection e.g. by producing neutralizing antibodies and cytotoxic T lymphocytes [Bowles et al. 1989]. For example, Figulla et al. [1995] demonstrated a better prognosis of enterovirus positive dilated cardiomyopathy compared to enterovirus negative dilated cardiomyopathy, which may include patients with an undetected, but inevitably fatal genetic etiology [Mestroni et al.1994]. This implies that some primarily enterovirus positive DCM patients, who cleared the myocardial infection by a sufficient immune response, never reach an "end-stage".

Recently, another heart muscle disorder, arrhythmogenic right ventricular cardiomyopathy, was found to be associated with enterovirus infections of the heart in a few patients [Giacca et al.1994, Heim et al. 1997]. Therefore, 6 patients with arrhythmogenic right ventricular cardiomyopathy were included in the present study. As the number of arrhythmogenic right ventricular cardiomyopathy patients was small, it was not primarily the goal of the present study to demonstrate the significance of myocardial enterovirus RNA in arrhythmogenic right ventricular cardiomyopathy. Nevertheless, it should be pointed out that 3 of 6 arrhythmogenic right ventricular cardiomyopathy patients were positive for enterovirus RNA in the EMB (Table I) whereas the 16 patients of control group B were all negative for enterovirus RNA in the myocardium ($P = 0.0023$, Chi-Square test). Moreover, the molecular and serological results of the enterovirus positive ARVC patients did not differ from the results obtained with enterovirus positive patients suffering from dilated cardiomyopathy. For example, CVB3 was found to be the predominant cardiotropic enterovirus in two of three ARVC patients compared to 6 of 7 DCM patients. CVB2 was demonstrated as myocardial pathogen by nucleic acid sequencing in the remaining two patients (one patient with ARVC and one patient with DCM). Similar to the enterovirus RNA positive DCM patients, neutralizing antibodies for the enterovirus serotype indicated by nucleic sequence analysis were demonstrated in all enterovirus RNA positive ARVC patients, whereas complement fixing antibodies were only detected in the ARVC patient with the shortest history duration (Table I). Although the number of ARVC patients was small, our data indicate similar virological and immunological mechanisms in enterovirus positive DCM and enterovirus positve ARVC. Future studies shall address the unresolved problem if individual genetic or environmental characteristics lead to either DCM or ARVC or a subclinical disease after an infection with a cadiotropic enterovirus.

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